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Award Number: DAMD17-01-1-0632

TITLE: Breast Cancer Escape from T Cell Rejection Mediated by Indoleamine 2,3-Dioxygenase

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REPORT DATE: July 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Burdent Paperwork Reduction Project (0704-0188) Washington, DC 20503

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4. TITLE AND SUBTITLE	5. FUNDING NUMBERS		
Breast Cancer Escape from T Cell Reje	ction DAMD17-01-1-0632		
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6. AUTHOR(S)			
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9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES	10. SPONSORING / MONITORING		
	AGENCY REPORT NUMBER		
U.S. Army Medical Research and Materiel Command			
Fort Detrick, Maryland 21702-5012			
11. SUPPLEMENTARY NOTES			
Report contains color			
12a. DISTRIBUTION / AVAILABILITY STATEMENT	12b. DISTRIBUTION CODI		
Distribution authorized to U.S. Government agencie			
(proprietary information, Jul 02). Other requests			
document shall be referred to U.S. Army Medical Re			
Materiel Command 504 Scott Street Fort Detrick			

13. ABSTRACT (Maximum 200 Words)

We performed research to support our hypothesis that human breast cancers have the capacity to evade a T cell mediated rejection at least in part by inducing the expression of an immunosuppressive enzyme called indoleamine 2,3-didoxygenase (IDO). Both of the objectives proposed in the original application were met. Using RT-PCR, we were able to demonstrate in a series of human breast tumors that IDO was expressed in the majority of these cancers. Furthermore, we made the interesting observation that the cells that invade breast tumors and tumor-draining lymph nodes that express IDO, as determined by immunohistochemical staining, are non-malignant mononuclear cells. This is supportive of our hypothesis that breast tumors induce the recruitment of IDO-expressing immunosuppresive antigen presenting cells as a mechanism of evasion of an immune mediated rejection. The second objective of developing a mixed autologous tumor cell/ lymph node cell model was also met. In this model a competitive inhibitor of IDO, 1-methyl-tryptophan, was ineffective in augmenting an anti-tumor T cell response. Future experiments will include a T cell priming strategy in addition to IDO inhibition.

14. SUBJECT TERMS Breast cancer, T cell,	15. NUMBER OF PAGES 11		
		-	16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Ommuted

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INTRODUCTION

In recent years there has been a resurgence in interest in the development of immunotherapy as an anti-cancer treatment modality. This has been driven by the demonstration that tumor associated antigens (TAA's) exist, and that T cells reactive to these TAA's are present in cancer patients. Furthermore, immunotherapeutic strategies have produced tumor regressions in clinical trials. However, the clinical response rates have been very low, so imrovements need to be made.

We have previously shown that a murine tumor which expresses a surface antigen (H-2K^b) that is recognized by 80% of the T cells in a T cell receptor (TCR) transgenic mouse (anti-H-2K^b TCR transgenic mouse) grows progressively when transplanted into these mice. We have found that the T cells present in the peripheral lymph nodes of tumor bearing mice are appropriately functional, however, when the T cells enter the tumor parenchyma they undergo apoptosis. We subsequently found that this was due to the expression of the immunosuppressive enzyme indoleamine 2, 3-dioxygenase within the tumors. Munn *et al* have demonstrated that IDO, through the catabolism of exogenous tryptophan, is the immunosuppressive enzyme that is responsible for the protection that fetuses have against rejection by allo-reactive T cells (1). T cells starved of exogenous tryptophan arrest in the cell cycle and eventually undergo apoptosis (2). We have found that the human breast carcinoma cell line (MCF-7) expresses IDO and that a competitive inhibitor of IDO, 1-methyl tryptophan, dramatically augments the T cell proliferative response to MCF-7 cells.

Patients who develop metastatic breast cancer often respond to hormonal therapy and chemotherapy, which results in a prolongation of survival. However, most patients' tumors eventually become refractory to these treatments. It is therefore important to develop alternative modalities of treatment. Immunotherapy is one such modality that holds promise. However, thus far, the efficiency of this approach in breast cancer patients has been very low. We have evidence to support the hypothesis that one reason that breast tumors escape an immune mediated rejection is through the production of the immunosuppressive enzyme IDO. Once proven in the pre-clinical setting, inhibitors of IDO can be incorporated into clinical trials which involve immunotherapy, which could increase the efficiency of this approach in breast cancer patients.

It is our hypothesis that one of the reasons that immunotherapy is ineffective in the treatment of breast cancer is because the immunosuppressive enzyme indoleamine 2, 3 dioxygenase (IDO) is produced within breast tumors. Furthermore, we hypothesize that inhibiting this enzyme may enhance T cell responsiveness to breast tumor cells.

BODY

Objective #1: A series of resected human breast tumors will be examined for the presence of apoptotic T cells, and for the expression of IDO.

Expression of IDO mRNA by Human Breast Cancers. We had previously shown that murine tumors have within them cells that express the immunosuppressive enzyme IDO. This lead us to the hypothesis that human breast cancers may evade a T cell mediated rejection by expressing IDO either directly or indirectly. In an attempt to determine if IDO is expressed within human breast tumors, we performed RT-PCR analysis on a series of 14 tumors. RNA was isolated by the Trizol method (Invitrogen) and used for RT-PCR with primers that were specific for the human IDO gene. CDNA was first produced by annealing random hexamer oligonucleotides (Invitrogen) to 10 µl of each RNA and then reverse transcribed with superscript II (Invitrogen). The reaction contained 10 ug of total RNA, 100 ng random hexamer oligonucleotides, 50mM Tris-HCl (pH 8.3), 75 mM KCl, 15 mM MgCl₂, 10 mM DTT, 10 mM each deoxyribonucleotide triphosphate and 500 units of superscript. Briefly, total RNA and random hexamer oligonucleotides were incubated at 65°C for 10 min and then placed on ice; the remaining components were then added, and the reactions were incubated at 37°C for 60 min and 70°C for 10 min. The cDNA generated was used as a template for PCR with primers specific for hIDO. PCR amplification was performed in a 50 µl reaction containing 3 µl of the reverse transcription reaction along with Taq DNA polymerase, 1X PCR buffer, 1.5 mM MgCl₂, 2mM each deoxyribonucleotide triphosphate, and 1µM each primer. PCR was carried out in a HYBAID omnigene thermocycler, and the conditions were one cycle (60°C for 3 min followed by initial denaturation at 94°C for 5 min), followed by 30 cycles of 94°C for 1 min, 55° for 1 min, and 72°C for 1 min. A final cycle was done at 94°C for 2 mins, 55°C for 2 mins, and 72°C for 10 mins. Ten µl of each reaction were run on a 2% agarose 1X Tris-borate-EDTA gel. Using this approach we found that the majority of the human breast tumors expressed IDO mRNA (Figure 1).



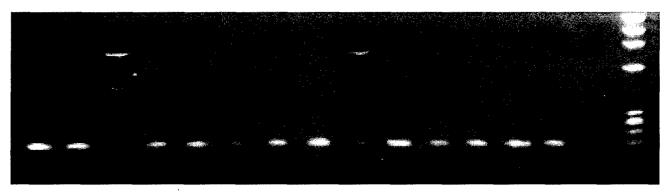


Fig. 1 IDO Expression of Human Breast Tumors Using RT-PCR Analysis. RNA was extracted from 14 human breast cancers and subjected to RT-PCR analysis. The PCR reactions were performed with primers that were specific for the human IDO gene (lanes 1-14). Lane 15 contained the negative control for the PCR reaction, and lane 16 contained the DNA size markers.

Immunohistochemical Analysis of Sentinel Lymph Nodes Resected from Breast Cancer Patients. In addition to showing that in our murine model that tumors escaped an immune mediated rejection at least in part through the induced expression of IDO, we were able to demonstrate that the relevant IDO-expressing cells were in fact antigen presenting cells. We were further able to demonstrate that these IDO-expressing antigen presenting cells were recruited to the draining lymph nodes of tumors, and were not present in non-draining lymph nodes of tumor-bearing mice. Since it is in the lymph nodes that T cells become activated, as naïve T cells do not have the capacity to extravasate into extra-nodal sites, this has lead to our model that tumors can recruit immunosuppressive antigen presenting cells (the IDO expressing cells) to tumor draining lymph nodes where there is present tumor associated antigens. T cells that are specific for these tumor associated antigens would then encounter these antigens in an immunosuppressive environment as the IDO-expressing antigen presenting cells are present.

In order to examine if this same phenomenon was occurring in human breast cancer patients, we examined the sentinel lymph nodes resected from breast cancer patients who underwent a clinically indicated procedure. The lymph nodes were fixed in formalin, embedded with paraffin, and tissue sections were cut for immunohistochemical staining. The paraffin sections were deparaffinized, treated for 8 min with proteinase K (Dako), and stained with rabbit anti-human IDO antibody [27] (10µg/ml in Tris buffered saline 0.05% Tween-20 and 10% goat serum). Detection was via secondary antibody conjugated to alkaline phosphatase (LSAB-rabbit kit, Dako) with Fast Red chromogen. Negative controls consisted of the anti-IDO antibody neutralized with >100-fold molar excess of the immunizing peptide used to generate the antibody. We found that the sentinel lymph nodes from several breast cancer patients were in fact infiltrated with non-malignant myeloid mononuclear cells that expressed IDO (Figure 2).

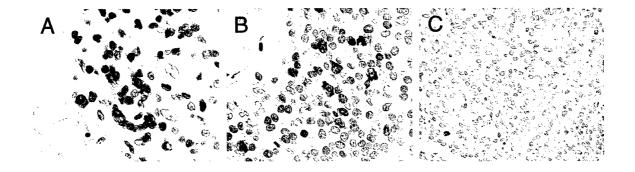


Fig. 2 IDO Expression in a Breast Cancer Sentinel Lymph Node. IDO-expressing cells (red immunohistochemical staining) are found in the leukocyte population infiltrating human breast cancer (A), and in the draining lymph nodes (B). In contrast, IDO-expressing cells were rare in normal lymphoid tissue (C).

Objective #2: The influence of IDO on T cell function will be determined in an autologous mixed lymphocyte/ breast cancer cell assay.

Human Mixed Autologous Tumor Cell/ Lymph Node Cell Model. In accordance with a University of South Florida IRB approved protocol, paired samples of autologous tumor and tumor-draining lymph nodes were isolated from resection specimens obtained from breast cancer patients who underwent a clinically indicated procedure at the H. Lee Moffitt Cancer Center and Research Institute, Tampa FL. The tumor and lymph nodes were disaggregated mechanically. The tumor tissue was further disaggregated by enzymatic digestion using an enzyme cocktail (2.0 mg/ml collagenase, 0.2 mg/ml protease, 600 U/ml Dnase). 5 x 10³ irradiated tumor cells (15,000 rads) and 4 x 10⁵ lymph node cells were combined in wells of a 96-well plate with or without 1 mM 1-methyl tryptophan which is a competitive inhibitor of IDO. At the end of the co-culture period, various assays of T cell function were performed.

ELISPOT Assays. Lymph node cells stimulated for seven days in the presence of irradiated tumor cells were subjected to ELISPOT analysis using kits from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions. The lymph node cells were transferred into the wells of an ELISPOT plate that had been pre-coated with a polyclonal antibody specific for human y-interferon or IL-2. Autologous tumor cell lysate was added to each well as a fresh source of tumor antigens. The lysate was generated by subjecting tumor cells to 6 cycles of freezing and thawing, 50 µl of lysate made from 2 x 10⁵ tumor cells was added to each well of the ELISPOT plates. After a 24 hour incubation in a humidified 37°C CO₂ incubator, the wells were washed and a biotinylated polyclonal antibody specific for human IL-2 or γ-interferon was added to the wells. Unbound biotinylated antibody was washed away and an alkaline phosphatasestreptavidin conjugate was added. Following 4 washes to remove unbound enzyme, a substrate solution (BCIP/NBT) was added. A blue-black colored precipitate formed and appeared as spots at the sites of cytokine localization. The number of spots were counted manually using a dissecting microscope. We did not observe an augmentation of an antitumor T cell response in this model with the sole manipulation of including a competitive inhibitor of IDO, 1-methyl tryptophan (Figures 3 and 4).

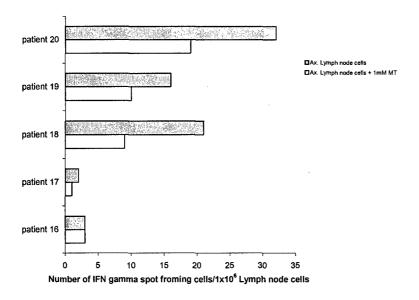


Fig. 3 Interferon-gamma Secreting T cells in a Mixed Autologous Tumor Cell/ Lymph Node Cell Assay. Human breast cancer cells were co-cultured with autologous lymph node cells for 7 days. At the end of this restimulation period the cells were subjected to an ELISPOT analysis using anti-IFN-gamma antibodies.

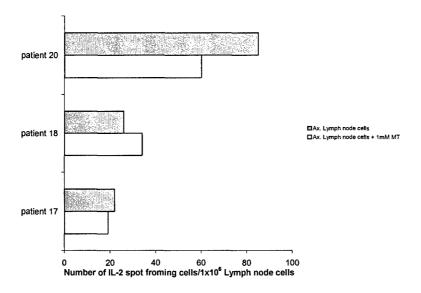


Fig. 4 Interleukin-2 Secreting T cells in a Mixed Autologous Tumor Cell/ Lymph Node Cell Assay. Human breast cancer cells were co-cultured with autologous lymph node cells for 7 days. At the end of this

restimulation period the cells were subjected to an ELISPOT analysis using anti-IL-2 antibodies.

KEY RESEARCH ACCOMPLISHMENTS

- 1. We were able to demonstrate that the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) is frequently expressed in human breast cancers.
- 2. We showed that IDO is expressed by non-malignant mononuclear cells that invade tumor-draining lymph nodes.
- 3. We were able to establish a unique model for the study of tumor induced suppression of anti-tumor T cell responses which was an *in vitro* mixed autologous tumors cell/lymph node cell model.
- 4. Inhibition of IDO in this model was not sufficient to reverse the tumor cell induced suppression of an anti-tumor T cell response.

REPORTABLE OUTCOMES

- 1. A manuscript reporting the data summarized in this report is in preparation.
- 2. The data summarized in this report will be used to support our NIH RAID program application for the development and production of 1-methyl tryptophan as a pharmacologic agent for use as a tumor vaccine augmentation strategy in the treatment of cancer patients.

CONCLUSIONS

It appears that the immunosuppressive enzyme IDO is expressed within a large fraction of human breast tumors. It also appears that the relevant IDO-expressing cells are non-malignant mononuclear cells. These data are similar to our findings in a murine model where IDO-expressing mononuclear cells infiltrate tumor-draining lymph nodes but not non-draining lymph nodes in the sane tumor-bearing animal. These data are consistent with our proposed model that tumors have the capacity to recruit immunosuppressive antigen presenting cells (IDO expressing cells) into tumor-draining lymph nodes. It is there that tumor antigen-specific T cells would encounter tumor associated antigens in a suppressive rather than activating environment due to the presence of these IDO-expressing cells.

In the human autologous mixed tumor cell/ lymph node cell model, adding a competitive inhibitor of IDO (1-methyl tryptophan) was not sufficient to prevent the tumor induced T cell suppression. There are several possible explanations for this preliminary observation. It may be that 1-methyl tryptophan is not an effective inhibitor of the IDO enzyme within the context of this model. It may also be that tumors possess multiple mechanisms whereby they evade a T cell mediated rejection, and that blocking just one of these mechanisms is not adequate. Finally it may be that there needs to be a T

cell priming event in this model in addition to inhibition of IDO. It may be that in future experiments where we will include a T cell priming strategy by adding into the model tumor vaccine cells, that the vaccine effect will be augmented by the addition of the inhibitor of IDO.

REPLY TO ATTENTION OF

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